AN IN VITRO SEROLOGICAL STUDY OF THE HETEROTRICH, BLEPHARISMA UNDULANS

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CHAPTER I

INTRODUCTION

This study represents an investigation of the *in vitro* effects of specific antibody on protozoan antigen, using *Blepharisma undulans* as the test organism.

The steps in this study were: (1) to prepare a vaccine from a pure culture of *Blepharisma undulans*; (2) to demonstrate an antibody response in guinea pigs, following inoculation with the *Blepharisma* vaccine; (3) to recover specific antibodies from serum of the inoculated guinea pigs; and (4) to test the serologic effects of these specific antibodies on the antigen determinant, *Blepharisma undulans*, using the low power dissecting microscope, the light microscope and the phase microscope as tools of observation.

A search of the literature revealed that no previous worker had used *Blepharisma undulans* as the test organism. Therefore data obtained in this study were interpreted by reference to the accumulated evidence in which other protozoans had been used as antigen determinants.

CHAPTER II

REVIEW OF THE LITERATURE

I. ANTIBODY FORMATION

An element, such as a protein, requires two proofs to be considered antigenic: first it must be a substance foreign to the organism in which it is introduced, and second, it must be capable of eliciting an antibody response. Its introduction into a host stimulates certain reactions which have been identified as typical antigen-antibody responses. The major reactions are agglutination, precipitation, flocculation and complement fixation.

If the presence of an antigen results in formation of an antibody then the antigen must react in some way with a substance in the body and perhaps alter it in some way. Furthermore, the process of immunization involves the building of a resistance to antigens, resulting in a large titer of antibodies. With the use of rabbits, tests have shown that during the immunization process there is a large increase in the blood serum protein.[1] Bjørneboe pointed out that this protein increase occurred in the gamma globulin fraction. He contended that this increase demonstrated a correlation between

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[1] M. Bjørneboe, "Specific Protein in Rabbit Antipneumococcal Serum and Its Relation to Increase in Serum Protein During Immunization," J. Immunology, XXXVII (1939), 201.

the antigens injected and the antibodies formed; the increase was due specifically to the formation of antibodies and also to an increase in the normal circulating gamma globulin.[1] Introduction of antigenic material into patients suffering from agammaglobulinemia resulted in no antibody response which was further evidence of the association between gamma globulin and the antibody.[2] Other workers studied this relationship with the use of electrophoretic techniques and showed that gamma globulin and antibodies showed the same pattern of migration.[3, 4] Other scientists have attempted to prove the exact relationship of antibodies and gamma globulin. Pauling suggested that antibodies were formed by a chemical alteration of normal gamma globulin; this normal gamma globulin differed from immune gamma globulin only in the sense that the latter had reacted with antigens.[5] Another theory, advanced by Haurowitz, indicated that antibodies were formed by the action

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[1] Ibid.

[2] G. H. Grant and W. D. Wallace, "Agammaglobulinemia," Lancet, II (1954), 671.

[3] K. Landsteiner, The Specificity of Serological Reactions (Cambridge, Massachusetts: Harvard University Press, 1945), p. 127.

[4] B. V. Jager, Principles of Internal Medicine (Philadelphia: Blakiston Company, 1950), p. 421.

[5] L. Pauling, Amino Acids and Proteins (Springfield, Illinois: Charles C. Thomas, 1951), p. 682.

of intermolecular forces of adjacent antigen molecules, reacting upon the precursors of normal gamma globulin.[1]

A further clarification of the close relationship between the antibodies and gamma globulin was investigated in studies which attempted to determine the exact location for formation of gamma globulin. Many workers indicated that the reticulo-endothelial system was a possible site for both gamma globulin formation and antibody formation.[2] More specifically lymphocytes as well as plasma cells were suggested as sites of antibody formation. The studies of Ortega and Mellors explained that gamma globulin was formed not only in the germinal centers of lymph nodules but also in the cytoplasm of immature and mature plasma cells.[3] The germinal center cells that synthesize gamma globulin were designated "intrinsic" cells to distinguish them from other medium to large sized lymphocytes that occurred elsewhere but did not participate in the production of gamma globulin. These intrinsic cells formed gamma globulin only when they were arranged in discrete aggregations. Ortega and Mellors further indicated that cells found to form gamma globulin appeared to be

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[1]F. Haurowitz, The Nature and Significance of Antibody Response (New York: Columbia University Press, 1953), pp. 2-10.

[2]H. Florey, Lectures on General Pathology (Philadelphia: W. B. Saunders Company, 1954), p. 100.

[3]L. G. Ortega and P. C. Mellors, "Cellular Sites of Formation of Gamma Globulin," Journal of Experimental Medicine, CVI (1957), 627.

identical with those cells found to form specific antibodies.[1] It was also suggested that each of the three morphologically distinct categories of cells that synthesized gamma globulin represented a separate response to a particular form of antigenic stimulation. These three sites of gamma globulin-antibody formation namely, germinal centers of lymph nodules, lymphyocytes, and plasma cells, indicated a close physical and perhaps chemical proximity between these two substances, gamma globulin and antibodies.

Other investigators carried on research pertaining specifically to theories of antibody formation. An account of these theories has been considered in a recent text by Holub and Jaroskova.[2] They stated that antibody formation might be a good example of the synthesis by the organism of a protein (the antibody) whose specific pattern was not determined wholly by the genetic machinery of the organism.[3]

Three hypothesis of antibody formation have been advanced. The first of these, the direct template theory, implied that the antigen was incorporated into the antibody-producing cell in such a way that it would act as a mold or

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[1] Ibid., p. 628.

[2] M. Holub and L. Jaroskova, Mechanisms of Antibody Formation (New York: Academic Press, 1960), p. 16.

[3] Ibid., p. 17.

template to impress a complementary pattern on globulin molecules synthesized by the cells. This theory involved three steps to antibody formation:

1. incorporation of the antigen into the plasma cell or intrinsic cell of the lymph nodule;
2. adherence of the antigen to the gamma globulin molecule causing an impression and
3. removal of the antigen from this gamma globulin molecule so it could impress the next gamma globulin molecule that came along.[1]

The indirect template theory differed from the first theory by assuming that all aspects of protein synthesis are under genetic control. The antigen will become incorporated into the cell and in some way become a part of the genome; all new templates would be produced by replication under genetic control.[2] The selection theory, indicated that genetic information could be developed by genetic processes only, never by an impact of the environment on the genome. This theory had the following features:

1. The phenomenon of immunity was based on the presence of clones of immunologically competent cells which can react with the corresponding antigenic determinant and/or produce cells which can synthesize and liberate antibodies.
2. The wide range of clones required to cover all possible antigenic patterns are developed in the course of embryonic differentiation.
3. Also during embryonic life clones which can react with antigenic determinants in its own body are eliminated; in this way information to differentiate self from non-self is obtained.[3]

The test of any antibody formation theory demands an explanation of the non-antigenicity by cells and proteins of the organism's

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[1] Ibid., p. 18. [2] Ibid. [3] Ibid.

own body; this third feature of the clonal selection theory attempts to answer this test.

At present a dual concept has been in favorable acceptance. This involved the combining of the clonal selection theory and the direct template hypothesis.[1] The clonal selection theory provided the genetic make-up for gamma globulin formation, while the direct template theory provided the environmental aspect of antibody formation.

II. STUDIES OF ANTIBODY FORMATION USING PROTOZOA AS THE ANTIGEN FACTOR

In this century protozoa have been used as experimental organisms in research particularly in relation to serological reactions. The main direction of immunological research with protozoa has been an effort to demonstrate resemblances or differences in certain strains, races and species. However, within these taxonomic studies has evolved information pertaining more specifically to the response of the protozoan to antiserum. The data in turn have been valuable in a consideration of the basic immunological reactions.

As early as 1902 Ledoux and Lebard studied the action of blood serum on paramecium.[2] By 1909 Rossle noted that the

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[1] Ibid., p. 20.

[2] Ralph Wichterman, The Biology of Paramecium (New York: Blakiston Company, Inc., 1953), p. 381.

antiserum of the rabbit or guinea pig made the pellicle of paramecia sticky.[1] Dilution of the immune sera up to ten times the normal sera led to intense paralysis of this protozoan. In 1911 Sellards studied immunity reactions, using the ameba as the experimental animal.[2] Takenouchi (1918) worked with different Infusoria, studying the cytological effects of normal and immune sera.[3] Paramecia were again studied by Rakieten (1928) using serologic reactions.[4] Amebae were studied as experimental animals in research on antigenic properties by Heathman (1932).[5] Robertson in 1934 used the flagellate, Boda caudatus, as the antigen determinant.[6] Robertson (1939) carried on further investigations, analyzing some of the properties of ciliates of the Glaucoma-Colpidium group.[7, 8] Following Robertson's studies, Bernhiemer and

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[1] Ibid. [2] Ibid.

[3] Takenouchi, "Cytologic Action of Normal and Immune Sera on Infusoria," J. Infectious Disease, XXIII (1918), 399.

[4] M. L. Rakieten, "Effects of Serological Systems on Paramecium," J. Immunology, XV (1928), 534.

[5] L. Heathman, "Studies of Antigenic Properties of Some Free Living and Pathogenic Amebas," American Journal of Hygiene, XVI (1932), 110.

[6] M. Robertson, "A Study of Reactions in Vitro of Certain Ciliates Belonging to the Glaucoma-Colpidium Group," Journal of Pathologic Bacteriology, VIII (1939), 310.

[7] M. Robertson, "An in Vitro Study of the Action of Immune Bodies Called Forth in the Blood of Rabbits by the Injection of the Flagellate Protozoan, Boda caudatus," J. Path. Bact., XXXVIII (1934), 374.

[8] M. Robertson, "An Analysis of Some of the Antigenic Properties of Ciliates of the Glaucoma-Colpidium Group," J. Path. Bact., VIII (1939), 330.

Harrison (1940) carried on some additional work with paramecia.[1] They demonstrated the presence of precipitin, complement-fixing and immobilizing antibodies in the serum of rabbits, after using paramecia as the antigen determinant. Kimball's work (1947) with paramecia was concerned with protozoan hereditary resistance to antiserum.[2] He found that when paramecia were placed in contact with a sublethal dose of antiserum over a twenty-four hour period and then subjected to higher concentrations of antisera, no protozoan immobilization occurred. Sonneborn's studies on paramecia provided exacting data in the field of protozoan taxonomy and immunology.[3, 4] Studies conducted by Loefer (1958) expanded on what has been called the mutual exclusion theory.[5] This theory states that only one antigen can be detected on the surface of a protozoan at any one time. He was able to demonstrate that

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[1]A. Bernhiemer and J. A. Harrison, "Antigen-Antibody Reactions in Paramecium, The Aurelia Group," J. Immunology, XXXIX (1940), 78.

[2]C. Kimball, "Heritable Resistance to Antiserum Produced by Antiserum and Trypsin in Paramecium Aurelia," Genetics, XXXII (1947), 93.

[3]T. M. Sonneborn, "Development and Inheritance of Serological Characters in Variety 1 of P. aurelia," Genetics, XXVIII (1943), 90.

[4]T. M. Sonneborn, "Acquired Immunity to Specific Antibody and Its Inheritance in P. aurelia," Proceedings of the Indiana Academy of Science, LII (1943), 191.

[5]J. B. Loefer, "Serological Types Among Thirty-One Strains of the Ciliated Protozoan, Tetrahymena Pyriformis," Journal of Protozoology, V (August, 1958), 210-212.

a given strain of protozoan, Tetrahymena pyriformis, appeared to have an inherent capacity to exhibit diverse antigens under different environmental conditions. Margolin (1959) continued the study related to this hypothesis.[1] He indicated that in regards to serological specificity a single gene for each serotype determined the specificity of the antigen and agreed with Loefer in that environment played an important role in determining antigenic specificity. Elliot and Byrd (1959) further validated Loefer's work.[2]

Since 1902 data have accumulated pertaining to serological reactions involving several species of protozoa. Throughout these investigations certain effects of the antiserum on the particular protozoan have been reported. A list of reactions which have been described follows. These are listed in a natural sequence starting from the protozoan's initial contact with the antiserum, to the ultimate death or recovery of the protozoan, as the case may be. All of these reactions are based on comparisons with reactions of the protozoans in normal sera.

The first sign of the antibody-antigen reaction was the gradual slowing of the organism in its swimming habits.

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[1] T. Margolin, "Immobilizing Antigens of Tetrahymena pyriformis," Journal of Protozoology, VI, No. 3 (August, 1959), 210-213.

[2] A. M. Elliot and J. R. Byrd, "Serotypes in Eight Varieties of Tetrahymena pyriformis," Journal of Protozoology, VI - Supplement (August, 1959), 18.

Over varying periods of time the organism eventually stopped the swimming motion and became immobile, as if paralyzed.[1, 2, 3, 4, 5, 6, 7] During this period of slowing, leading to immobilization other phenomena have been described. Most of the investigators reported accumulations of an exudate material, a sticky substance, which adhered to the cilia and pellicle.[8, 9, 10] These microscopic drops seemed to form near the end of the cilia. Another experimenter noted a slight shrinkage of the pellicle.[11] Others reported that clear blisters formed on the surface of the protozoa. Later (following formation of blisters) the body form altered and a bump

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[1]Wichterman, op. cit., p. 392.

[2]Robertson, "A Study of Reactions in Vitro of Certain Ciliates Belonging to the Glaucoma-Colpidium Group," p. 318.

[3]Bernhiemer, op. cit., p. 82.

[4]Sonneborn, loc. cit. [5]Loefer, loc. cit.

[6]Margolin, loc. cit. [7]Elliot, loc. cit.

[8]Robertson, "An in Vitro Study of the Action of Immune Bodies, Called Forth in the Blood of Rabbits by the Injection of the Flagellate Protozoan," loc. cit.

[9]Bernhiemer, loc. cit. [10]Sonneborn, loc. cit.

[11]Robertson, "A Study of Reactions in Vitro of Certain Ciliates Belonging to the Glaucoma-Colpidium Group," loc. cit.

appeared near the posterior of the protozoan on the dorsal side. Immobilization was usually followed by plasmolysis of the cell.[1, 2, 3, 4, 5] Not all species of protozoa reacted exactly the same to the antisera; this seemed to indicate that some of the organisms might be more sensitive as test antigens.[6] Kimball[7] and Sonneborn[8] both have reported cases of protozoan ability to build up a resistance to specific antisera; this immunity was passed through several generations, but only by the mechanism of fission.

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[1]Bernhiemer, loc. cit. [2]Sonneborn, loc. cit.

[3]Loefer, loc. cit. [4]Margolin, loc. cit.

[5]Elliot, loc. cit. [6]Bernhiemer, op. cit., p. 76.

[7]Kimball, loc. cit. [8]Sonneborn, loc. cit.

CHAPTER III

STRUCTURAL CHARACTERISTICS AND NORMAL SWIMMING HABITS OF BLEPHARISMA UNDULANS

I. CLASSIFICATION[1]

Although there are many ways of classifying Blepharisma undulans, the following classifications are accepted by many protozoologists:

Phylum: Protozoa
Class: Ciliata
Subclass: Spirotricha
Family: Plagiotomidae
Order: Heterotricha
Genus: Blepharisma
Species: undulans

II. STRUCTURAL CHARACTERISTICS OF BLEPHARISMA UNDULANS

Blepharisma undulans, a pink-colored heterotrich varies from 150 to 300 microns in length. Its body is elongated, coming to a point on its anterior end, the point slanting a little to the left. The posterior end is rounded while the sides are convex dorsally and subsequently concave ventrally, becoming less concave toward the anterior end.

The cilia can be classed into three groups according to

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[1]G. Calkins, Biology of the Protozoa (New York: Lea and Febiger, 1926), p. 386.

their relative size and location. Somatic ciliature is short and in parallel rows.[1] The peristome stretches out from the anterior extremity just about reaching the midline; it averages about two-fifths of the total body length. The peristome is bounded on the left by an adoral band of cilia, longer and thicker than the body cilia; it is this adoral zone that characterizes the subclass, Spirotricha and the order, Heterotricha.[2] The adoral zone is bounded on the right side by a membranelle which forms an undulating membrane, occupying between one-half and two-thirds of the right peristomal border.[3, 4, 5, 6, 7]

The macronucleus is located close to the midline and appears to be in two portions when stained with methyl green

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[1]D. L. MacKinnon and R. S. Hawes, An Introduction to the Protozoa (Oxford: Clarendon Press, 1961), pp. 344-345.

[2]Ibid.

[3]Saville-Kent, Manual of the Infusoria (London: David Bogue, 1881-1882), II, 583-585.

[4]E. Penard, Infusoires, Etude sur les Infusoires d'eau Douce (Geneve: Georg and cie, 1922), p. 204.

[5]A. Kahl, Wimpertiere oder Ciliata (Infusoria). In Dahls Die Tierwelt Deutschlands und der angranzenden Meieresteille (Fischer, Jena, 1930), p. 924.

[6]F. Doflein, Lehrubch der Protozoenkunde (Fortgesetzt: von Edward Reichenow, 1949), p. 1127.

[7]G. N. Calkins, Protozoology (New York: Lea and Febiger, 1909), p. 54.

dye. One portion is located in the anterior one-half of the organism and the other in the posterior one-half.[1] These two parts are connected by a cord-like funiculus.[2] Although the micronuclei do not stain as clearly as the macronucleus, three to four of them were counted in several of the organisms.[3]

III. NORMAL SWIMMING HABITS

Blepharisma undulans is a comparatively sedentary organism. Its normal swimming movements are restricted to those required for food gathering such as slow crawling movements along the bottom and sides of the container. These movements are occasionally accompanied by bursts of rapid movement. However, if the organism is agitated, for example by being suctioned in and out of a pipette, its normal swimming movements are accelerated. The relatively short, undulating membrane can account in part for the rolling movements observed in all normal swimming by the organism. During agitation the avoiding reaction, that is, movement toward and sudden movements away from objects in the solution, is decidedly increased. After a short interval the agitated organism becomes sedentary, resorting to swimming habits used in gathering food.

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[1] R. R. Kudo, Protozoology (Springfield, Illinois: Charles Thomas, 1954), pp. 802-803.

[2] Saville-Kent, loc. cit. [3] Penard, loc. cit.

CHAPTER IV

MATERIALS AND METHODS

I. ISOLATION OF BLEPHARISMA UNDULANS

Blepharisma undulans was isolated from a mixed culture of protozoa, containing not only Blepharisma undulans, but also Colpidium sp. and Euplotes sp. By suction of culture fluid into a micropipette, attempts were made to capture one Blepharisma undulans from the mixed culture. Once captured, the organism was transferred to a spot plate depression which contained one ml. of sterile saline. The organism was passed through a series of not less than seven saline washes. By micropipette the protozoan was transferred from one depression to the next. After it had passed through the wash series, Blepharisma undulans was transferred to a test tube, three inches in length, which contained five ml. of water and some ground particles of dried navy beans. These beans served as a suitable media for growth and reproduction of Blepharisma undulans. All equipment utilized in isolation and vaccine preparation of B. undulans was sterilized by the use of an autoclave which was set at fifteen pounds of pressure for a period of fifteen minutes.

Several cultures were started in this manner. Each culture was pure in the sense that it contained only the single organism, B. undulans, and no other protozoa, although

bacteria flourished in these bean cultures also. After a period of sixteen days the cultures were consolidated into a single beaker, making a total volume of fifty ml.

With a stirring rod the organisms were distributed throughout the culture. Immediately after agitation a 0.5 ml. sample was pipetted from the culture. This 0.5 ml. volume was diluted to twenty times its original volume. From this dilution a 0.5 ml. sample was taken and with the aid of the dissecting scope (20x magnification), the number of organisms present in this volume were determined. Ten different counts were made to obtain an average number of organisms in the diluted sample. This average number taken times the dilution factor (twenty) indicated the number of protozoa in the original 0.5 ml. volume obtained from the original fifty ml. volume. This figure taken times two indicated the number of organisms in one ml. of the original culture; the number of organisms was estimated to be 3000/ml.

II. VACCINE PREPARATION

Standard (three inches long) vaccine vials were properly fitted with a rubber cap which, when snapped over the tube, provided an air tight compartment within the tube. These vaccine tubes could hold ten ml. of solution.

The protozoan culture was concentrated to a 25 ml. volume by spinning down the organisms in a centrifuge at a speed of 500 r.p.m. per minute, followed by withdrawing some

of the culture solution by pipette. The protozoa were washed free of culture media with sterile saline. With the aid of a pipette the organisms were transferred to a five inch long tissue grinder which consisted of a pestle and a heavy glass tube into which the pestle fit tightly. By vigorous grinding, the cells were broken up. The pestle and tube were washed thoroughly with a 0.85 per cent sterile saline solution. The wash freed the organisms from the grinder and also was added to provide a total volume of twenty-five ml. This solution, consisting of disrupted cells, cilia and 0.85 per cent saline, was divided between the three serological vaccine tubes. These tubes were plugged tightly with the rubber caps and immediately placed in a hot water bath (60° Centigrade) for a period of three hours after which they were refrigerated (3° Centigrade) until inoculation. Hereafter this solution will be referred to as a vaccine.

III. IMMUNIZATION PROCEDURE

The test host organisms were guinea pigs, Cavia cobaya, obtained from Diamond Laboratories, Des Moines, Iowa. All animals were fed a diet of rabbit pellets and lettuce. Their water contained .09 per cent of ascorbic acid, since the normal flora of the guinea pig intestine does not aid in formation of this substance.

Using a one ml. syringe, five guinea pigs were inoculated intraperitoneally with the protozoan vaccine. The guinea pigs

were immunized using the following inoculation schedule:

Day	1	3	6	10	17
ml. of vaccine	1.0	1.0	1.0	1.0	1.0

Two guinea pigs were used as controls, receiving no inoculations.

Two weeks after the final inoculations the guinea pigs were bled by heart puncture, using a 10 ml. syringe. The whole blood was immediately transferred from the syringe to appropriately labelled six inch long test tubes. The tubes were plugged with cotton and refrigerated at 3° Centigrade overnight.

By ringing the clotted blood within the tubes with a sterile needle, separation of the clotted portion from the serum was facilitated. The clot no longer adhered to the test tubes and settled to the bottom, leaving the free serum. Some hemolysis occurred, since the serum was tinted pink.

The serum was removed from these test tubes with a serological pipette and transferred to six inch long serology tubes. The serum was placed in a freezer (-1° Centigrade) until tests were performed. This serum hereafter will be referred to as antiserum or immune serum.

IV. TECHNIQUES USED TO DETERMINE EFFECTS OF ANTISERUM ON *B. UNDULANS*

During vaccine preparation and antiserum production, a culture of *B. undulans* was maintained in the navy bean culture

media. The strength of the antibody titer from each of the five guinea pigs was determined by dilution. Drops of culture, containing the Blepharisma organism were added to each of the antisera to obtain dilutions of 1:3, 1:6, and 1:10. The highest titer was quantitatively defined as that dilution in which some of the organisms survived over a time interval of two and one-half hours.

To determine the effects of the antisera on B. undulans four distinct procedures were employed. To one drop (0.05 ml) of serum (immune or normal) was added one drop (0.05 ml.) of culture containing B. undulans. All drops mentioned hereafter will be 0.05 ml. in volume. Drops were placed in depressions of spot plates; observations were made with the dissecting scope. With the aid of stop watch, the number of minutes which were required for complete immobilization of all of the protozoa to occur was determined. This time was recorded to the nearest minute. Complete immobilization refers to the absence of any ciliary movement. Each depression, containing antiserum and culture, was observed continuously for fifteen minutes to determine if complete immobilization occurred within this time interval.

A second group of tests was performed to ascertain the extent of the effects of immune serum on the protozoan. In this case one drop of culture, two drops of tap water and three drops of serum were placed into depressions of spot plates. This constituted a one to one dilution; the water

was added to avoid effects of evaporation. Again observations were made using the dissecting scope. The organisms were studied for a period of five hours at intervals of thirty minutes, one, two, three, four and five hours.

The more detailed aspects of the effects of the immune serum on B. undulans were arrived at in a third study. A drop of serum (immune or control) and a drop of culture were placed on a clean, dry microscope slide. Observations were made using the light microscope (magnifications of 100x and 430x). Changes in the organisms in contact with the immune or control sera, either leading up to immobilization or following it, were observed in this procedure. One organism was studied at a time; ten organisms were studied for each of the control and immune sera.

In the fourth series, tests were made to determine the presence of a possible surface or subsurface phenomena, related to the immobilization reaction. The phase microscope was used for these observations (bright and dark phase contrasts at 920x magnification). Fifty separate observations were made, viewing several hundred organisms.

CHAPTER V

RESULTS AND DISCUSSION

I. STRENGTH OF TITER DETERMINED BY DILUTION OF SERUM

Data related to the relative strength of the various antisera will be found in Table I. In a 1:3 dilution of antisera from all five guinea pigs, Blepharisma were immobilized and plasmolysis promptly occurred. In the 1:6 dilution the organisms at first appeared slow and then at the end of a period of two hours, normal activity had been regained. On the 1:10 dilution only in the case of antisera from guinea pig five was a slowing recorded; all others showed no effects at all. The fifth antiserum may have had a slightly higher titer than the other four. Since no ill effects were observed in antisera I-IV at the 1:10 dilution, this volume served a definition of titer strength. A relatively low titer was obtained in these experiments.

Rakieten has reported paralyzing effects at dilutions up to 1:2000.[1] This method of dilution was an indirect means of determining the strength of titer: the greater the dilution required to halt the effects of the immune serum, the higher the antibody titer.

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[1]Rakieten, op. cit., pp. 527-537.

TABLE I

THE RELATIVE STRENGTH OF ANTIBODY TITER, DETERMINED BY IMMOBILIZATION OF BLEPHARISMA UNDULANS IN SEVERAL DILUTIONS OF ANTISERUM

Samples	Dilution 1:3	Dilution 1:6	Dilution 1:10
Antiserum Guinea pig I	****	*-O	O
Antiserum Guinea pig II	****	*-O	O
Antiserum Guinea pig III	****	*-O	O
Antiserum Guinea pig IV	****	*-O	O
Antiserum Guinea pig V	****	****	*-O

KEY - **** Complete immobilization

*-O Initial slowing followed by a return to normal behavior

O Normal swimming behavior

II. IMMOBILIZATION TIMES

The length of time in which observations were recorded on both the control sera and experimental antisera was fifteen minutes. Since all Blepharisma in the controls were alive at the end of this fifteen minute interval, this was the time used to compare with the average immobilization time for each of the five experimental sera (see Tables II-VIII). By inspection it was concluded that there was a variation between the immobilization time for each of the immune sera as compared to the control sera. A "t" test was utilized to determine the significance of difference between the immobilization time of each of the antisera as compared to the mean immobilization time for all five antisera combined (4.7 minutes).

TABLE II

A "t" TEST COMPARISON OF THE INDIVIDUAL MEAN IMMOBILIZATION TIMES TO THE OVER-ALL MEAN IMMOBILIZATION TIME

Antiserum	Mean Time (minutes)	"t" Value	Probability
I	5.1	.52	.6P .7
II	5.1	.52	.6P .7
III	4.7	0	0
IV	4.1	1.20	.2P .3
V	4.5	.76	.4P .5

TABLE III

IMMOBILIZATION TIMES OF *BLEPHARISMA UNDULANS* IN 1:1 DILUTION OF ANTISERUM FROM GUINEA PIG I

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	0
1	0	0	0	*	*	0	0	0	*
2	*	*	*	**	**	0	*	*	*
3	*	**	*	***	***	*	**	**	***
4	*	****	*	****	****	***	***	****	***
5	**		**			****	***		****
6	***		***				****		
7	****		***						
8			****						
9									
10									
15									

KEY - 0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

TABLE IV

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS IN 1:1 DILUTION OF ANTISERUM FROM GUINEA PIG II

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	0
1	*	**	0	0	0	0	0	0	0
2	**	***	0	0	0	0	0	**	***
3	**	***	0	0	*	0	0	****	***
4	****	****	**	*	**	0	0		****
5			**	**	***	0	0		
6			***	****	****	0	***		
7			****			0	****		
8						0			
9						0			
10						0			
15						0			

KEY
0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

TABLE V

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS IN 1:1 DILUTION OF ANTISERUM FROM GUINEA PIG III

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	*
1	*	*	*	*	*	*	*	*	**
2	**	**	**	*	**	**	*	*	****
3	***	****	***	**	***	**	*	**	
4	****		****	****	****	**	**	***	
5						***	**	****	
6						****	***		
7							***		
8							***		
9							****		
10									
15									

KEY

0	Normal Behavior
*	Slowing
**	Movement Markedly Slowed
***	Almost Total Paralysis
****	Complete Immobilization

Mean 15. minutes

TABLE VI

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS IN 1:1 DILUTION OF ANTISERUM FROM GUINEA PIG IV

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	*	0	*	*	*	*	*	*	*
1	*	*	*	**	**	**	***	*	*
2	**	**	**	***	***	**	***	**	**
3	****	***	***	****	****	***	***	***	***
4		****	****			****	***	***	***
5							****	***	****
6								****	
7									
8									
9									
10									
15									

KEY
0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

TABLE VII

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS IN 1:1 DILUTION OF ANTISERUM FROM GUINEA PIG V

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	0
1	*	0	0	0	*	*	*	*	0
2	*	*	*	*	**	*	**	**	*
3	***	**	**	**	***	**	***	***	**
4	****	***	***	****	****	***	****	****	***
5		****	****			****			****
6									
7									
8									
9									
10									
15									

KEY
0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

By summing the mean immobilization times listed in Table II and dividing this figure by five, the total average time (4.7 minutes) required for complete immobilization of the organism to take place was thus computed. In Table IV one test indicated the organisms had remained alive for a period of fifteen minutes. This interval of time was statistically invalidated and discarded when compared with the other tests in this series; for this reason antiserum II had a mean immobilization of 5.1 minutes. Tables VII and VIII indicated that all organisms were alive at fifteen minutes after contact with normal serum. However, some slowing of the B. undulans was observed toward the end of this fifteen minute interval.

In reference to the "t" test and more specifically to antisera of guinea pig I as an example, a probability between 0.6 and 0.7 was computed. This probability indicated that sixty times out of one hundred times a difference of 0.4 minutes could occur. Only when the probability values, as determined by the "t" test, are within the range of .05-.001 is a significant difference indicated in the two figures being compared. Each of the five guinea pig antisera were compared to the total average time, 4.7 minutes. In the antiserum from guinea pig IV the greatest variation (.6) from the total average time was indicated. The probability that this difference would occur within the samples being compared was between .20 and .30. This value approached the .05 level but

TABLE VIII

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS
IN 1:1 DILUTION OF SERUM*
FROM GUINEA PIG I

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	0
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
15	0	*	0	*	*	0	0	0	0

*Indicates normal serum.

KEY
0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

TABLE IX

IMMOBILIZATION TIMES OF BLEPHARISMA UNDULANS
IN 1:1 DILUTION OF SERUM*
FROM GUINEA PIG II

Minutes	Individual Spot Plate Tests								
	1	2	3	4	5	6	7	8	9
0.5	0	0	0	0	0	0	0	0	0
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
15	*	0	0	0	*	0	0	0	*

*Indicates normal serum.

KEY
0 Normal Behavior
* Slowing
** Movement Markedly Slowed
*** Almost Total Paralysis
**** Complete Immobilization

Mean 15. minutes

did not represent a really significant difference. Had one of the five mean times fallen within the .05 limit, the "t" test would statistically imply that only five out of one hundred times such a difference would occur within the framework of the data under consideration. Such a low probability value in a "t" test analysis would indicate statistically unreliable data. However, little statistical difference exists between the mean over-all immobilization time and the individual immobilization times. The fact that little difference was indicated illustrates the similar effects that each of the antisera have on the Blepharisma organism, that is to say, almost all organisms were paralyzed within a relatively close time to the over-all mean immobilization time (4.7 minutes).

Within each depression of a particular spot plate which contained one of the antisera and the organism, not all protozoa were immobilized simultaneously. Only when the last organism in the depression had stopped moving was the time recorded. The absence of an identical reaction of all organisms within even one depression could be due to what Margolin calls the split reaction; that is some animals responded in his experiments while others did not under the same experimental conditions.[1] Also Bernhiemer and Harrison indicated in their work that some paramecia were more sensitive as test antigens than were others.[2] Other variables might explain

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[1]Margolin, op. cit., p. 213.

[2]Bernhiemer and Harrison, op. cit., p. 80.

differences reported between each of the five antisera. Differences in the experimental animals, such as size and physiological conditions of the guinea pigs during inoculation, are examples. All five animals may not have built up a titer of antibodies to the same level; at any rate a variance in the titer between animals at the time of bleeding is a definite possibility. Some inconstancy can be attributed to variation in technique during inovulations and bleeding.

Studies conducted over longer periods of time than were done so in this research illustrated the effects of normal serum on paramecia.[1] For example unless sodium chloride was present in the serum in the proper concentration, paramecia were immobilized; in normal serum however this phenomenon was not observed for several hours (usually about twenty-four hours).

III. FIVE HOUR OBSERVATIONS

The results of observations on the two control and five experimental sera for this section are indicated in Table X. Nine separate observations for each control and experimental series were studied. All of the organisms in each of the immune sera were immobile at the end of the first half hour. Further observations at intervals of one, two, three, four, and five hours revealed complete immobilization and lysis in all

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[1] Takenouchi, op. cit., p. 399.

TABLE X

IMMOBILIZING EFFECTS OF GUINEA PIG ANTISERA ON BLEPHARISMA UNDULANS OVER FIVE HOURS

Hours	Control		Immune Serum				
	Guinea Pig 1	Guinea Pig 2	Guinea Pig 1	Guinea Pig 2	Guinea Pig 3	Guinea Pig 4	Guinea Pig 5
0.5	*	0	****	****	****	****	****
1	**	0					
2	0	0					
3	0	0					
4	0	0					
5	0	0					

KEY

0	Normal Behavior
*	Slowing
**	Movement Markedly Slowed
***	Almost Total Paralysis
****	Complete Immobilization

five cases of the antisera solution. In the five of the nine solutions in the depressions concerned with Control I some initial slowing in B. undulans swimming habits was noted at one-half hour and again at the end of one hour. Normal activity of the protozoan was recorded at the time intervals thereafter for the Control I Study. In Control II organisms in all of the nine solutions performed normally in their swimming habits throughout the five hours.

The immobilization of the protozoa noted at the end of the first half hour of contact with antisera was an irreversible phenomenon as no temporary paralysis was noted. It would be assumed that immobilization was the result of antigen-antibody combination. Immobilization was inevitably followed by cell destruction; no cells were observed in the immune sera preparations after one hour. Loefer's investigation indicated that in a course of several hours (usually twenty four) organisms in the 1:40 and 1:80 dilutions recovered to normal activity.[1] In higher concentrations of antisera the organisms did not recover. This phase of the study on B. undulans supported Loefer's work, since no recovery was observed in the concentrated antisera preparations.

The normal swimming activities reported over a five hour interval in the controls further substantiated some definite difference in the chemical components of the control sera and the immune sera.

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[1]Loefer, op. cit., p. 211.

IV. LIGHT MICROSCOPE OBSERVATIONS OF THE IMMOBILIZATION PHENOMENA

Although there was some variation in response of B. undulans to the various antisera, the following phenomena were considered as typical responses. Within thirty seconds a slowing in the beats of both somatic and oral ciliature was noted; however in this short interval no malfunction in the rhythmic beat of the cilia was noted. Changes in the undulating membrane could not be perceived. These cilia beat even more slowly at the end of fifty seconds. Within this time interval many organisms rotated on their posterior axis both in a clockwise and counter-clockwise manner, a condition not observed in the normal swimming habits of B. undulans. Following initial contact of a protozoan with the immune sera, several varied phenomena took place within a one to three minute interval. Oral and somatic ciliature lost their rhythmic geat, moving spasmodically and at a slower rate than previously observed. The first group of cilia to become immobile were the short, somatic cilia; the oral membranelle of the adoral zone continued to beat for as long as two to three minutes after the somatic ciliature had ceased movement. Within this time interval droplets of a material (exudate) began to appear on both the somatic and oral cilia. The amount of this exudate increased with time; in some cases organisms could be observed swimming sluggishly with particles of this exudate trailing behind them. This material appeared to have

the effect of sticking the somatic cilia together, resulting in a random crisscrossing of these cilia. Also during this one through three minute interval some body twisting and contortion, atypical to normal movement of the organism was observed. Finally the adoral cilia (membranelle) ceased beating, and the cell became completely immobile. Following this total ciliary paralysis, the cytoplasm within the cell membrane appeared to move vigorously; no blebs or evaginations of the membrane were observed. However the cell membrane usually ruptured, following total paralysis of the cilia and cytoplasm would then flow into the surrounding fluid (plasmolysis). Rupturing of the membrane occurred most often in the buccal region, although less frequent breaks would occur toward the posterior aspect of the protozoan.

The original slowing mechanism of ciliary movement must have been due to some substance in the antiserum which was presumably an antibody specific for antigens of B. undulans. This could be postulated as the primary cause for slowing of ciliary movement. Since ciliary movement was affected noticeably before any other changes, such as lysis took place, the antigen most likely involved was a ciliary antigen. Lwoff in an analysis of Sonneborn's work,[1] made the following comments,

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[1]T. M. Sonneborn, "Symposium on Plasma Genes, Genes, and Characters in Paramecium aurelia," American Naturalist, LXXXII (1948), 26-34.

concerning the paralyzing effect of the antisera on cilia, as reported in Sonneborn's work: he contended that the responsible antigens would be ciliary antigens.[1] He concluded that since cilia are produced by the kinetosomes, these bodies could have a share of the responsibility in the antigen-antibody phenomenon in Sonneborn's work. He concluded that a substance analogous to the ciliary antigen could exist in the kinetosome.

However, Preer and Preer in a more recent investigation, using paramecia, reported the presence of antigens that could be classified as immobilizing antigens.[2] They indicated that the immobilizing antigens were determined genetically by a remarkable system of heredity, involving both cytoplasmic and Mendelian factors. Four antigens were isolated and studied. The fourth of these appeared to be the best antibody producer of the cell; it was determined to be the immobilization antigen of that particular strain of paramecia. They concluded that a large proportion of this antigen was associated with the body while another larger portion was found in cilia; little or none was located in trichocysts.

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[1]Andre Lwoff, Problems of Morphogenesis in Ciliates (New York: John Wiley & Sons, 1950), pp. 46-47.

[2]J. Preer and L. P. Preer, "Gen Diffusion Studies on the Antigens of Isolated Cellular Components of Paramecium," Journal of Protozoology, XI (February, 1959), 88-99.

The immobilization of somatic cilia, followed by the oral cilia at a later time supported work performed particularly by Loefer.[1] It may be postulated that since the somatic cilia are shorter and finer than the adoral cilia, that paralysis was merely a function of size, the smaller cilia being affected before the larger. No evidence other than the relative sizes of the cilia can support this postulate.

The presence of the exudate, as droplets, would appear to be related to a phase of the antigen-antibody combination, although no direct evidence indicated this. Since no such exudate was formed on the organisms in control sera, its presence must be attributed to a substance not present in normal serum, again presumably the antibody. Robertson noted in an investigation of the Glaucoma-Colpidium group that the exudate not only interfered with ciliary movement but caused organisms to stick together in a random fashion.[2] A decrease in the movements of cilia mechanically, if in no other manner, was brought on by the presence of this exudate.

Loefer performed more precise investigations on the nature of an exudate similar to the one he had observed in his Tetrahymena sp. studies.[3] By selective ultracentrifugation a gelatinous material was separated from the organisms with which

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[1]Loefer, op. cit., p. 213.

[2]Robertson, op. cit., p. 320.

[3]Loefer, loc. cit.

Loefer was working. He added this material to a solution of Tetrahymena; then antisera was added to the culture. The presence of the gelatinous material increased the time of maximal immobilization, as well as protecting the organisms against lysis. The protective effect did not appear to be related to the specific neutralization of antibodies or to the particular character of the ciliary antigen. This protective substance may or may not be related to the exudate extruded during protozoa contact with specific antisera. Loefer hypothesized that the exudate might not be an emission, combining with antiserum, but a protective mechanism.

Loefer[1] and Robertson[2] both observed a shell completely surrounding and adjacent to the pellicle of the protozoa they used as test organisms in antiserum. These protozoa escaped from the exudate shells and the remains of these shells could be clearly observed with imprints of cilia noticeable. No shell was observed on or adjacent to the pellicle of B. undulans and no empty shells or fragments were observed.

Rupture of the cell membrane appeared to be an indirect consequence of the immobilization of the cell cilia. No active feeding was observed. The inactivity of the cilia might have resulted in an upset in the delicate balance on the interior of the cell. Increased cytoplasmic flow in comparison to the

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[1] Ibid. [2] Robertson, loc. cit.

normal flow could possibly be looked upon as an effort by the organism to compensate for a change in general cell activity, or it could be postulated that this increased flow was an initial step in plasmolysis. No evidence readily supports these hypotheses.

V. PHASE MICROSCOPE INVESTIGATIONS

The immobilization phenomenon and exudate formation observed using the phase microscope, did not validate the actual presence of a surface phenomenon. However some new data, not observed in light microscope studies, were obtained with the aid of the phase microscope. Using both the light and dark phases of the microscope, observations were made on organisms in all five antisera. Attention was primarily paid to internal cellular activity, leading up to lysis of the cell. As in the case of light microscope observations cytoplasmic flow seemed to increase considerably. However, there was no obvious migration of intracellular granules and vacuoles toward the membrane. In a few cases after total immobilization of cilia was noted, movement of adoral cilia was observed. This movement lasted approximately ten seconds and was abruptly followed by rupture of the cell in the oral region. The origin of the exudate before its appearance on the cilia could not be determined by observations using the phase microscope.

The later movement of the adoral cilia was noted with the phase scope, due to its light and dark contrast, a contrast

not provided with the use of the light microscope. The movement was only slightly perceptible, occurring as long as three minutes after total immobilization of cilia but lasting for only a short duration. The membranellar movement might have been a factor, determining location of rupture of the membrane. This rupture occurred at the oral region in every case in which the adoral cilia moved after total paralysis had been previously observed. However many organisms that did not demonstrate a later ciliar movement in the oral region continued to plasmolyse through this region. This "latent" adoral ciliar movement may have been nothing other than a delayed final contraction of this ciliature.

CHAPTER VI

SUMMARY AND CONCLUSION

I. SUMMARY

This study represents an investigation of the *in vitro* effects of specific antibody on protozoan antigen, using *Blepharisma undulans* as the test organism. A pure culture of *Blepharisma undulans* was prepared containing approximately 3,000 organisms per ml. of culture, as estimated by a dilution method. From this culture a vaccine was prepared using standard procedures. A schedule of injections of this vaccine intraperitoneally into five guinea pigs was followed to build a high titer of antibodies from the foreign antigen, *B. undulans*. Two weeks after the final inoculation, whole blood was collected from the five guinea pigs receiving inoculations and from two guinea pigs, used as controls, receiving no inoculations. Both immune and control sera were separated from the whole blood and refrigerated.

The effects of the control and immune sera on *B. undulans* were investigated in five separate studies. Study I indicated that the organisms were not affected by immune sera at a dilution of 1:10. Dilutions of 1:3 and 1:6 resulted in immobilization and lysis of *B. undulans*. Study II was an investigation of the time required to completely immobilize the organism in the presence of the antisera. Immobilization

was defined as complete absence of ciliary movement. The mean immobilizing time for the five experimentals was 4.7 minutes, while in the control sera organisms were still actively mobile after fifteen minutes. Statistically "t" tests computations indicated that there was no significant difference between the over-all mean immobilization time, 4.7 minutes, and the mean times for each of the five antisera. Differences between the controls and the experimentals had to be due to the presence of a substance in the antisera which was not present in the control sera; presumably this substance was the antibody specific for B. undulans.

Study III was an investigation to determine if the immobilization phenomenon was only temporary. At hour intervals solutions containing the organism and the sera were studied to see if B. undulans would return to its normal swimming habits after immobilization. All organisms in the 1:1 antisera-culture dilution were immobilized with the first observed time interval. At one, two, three, four, and five hours no recuperation from the immobilized state was observed. Instead all organisms in the antisera had been lysed and had completely disappeared after the one hour observation. In Control sera the organisms were actively mobile after five hours.

Study IV was concerned with light microscope observation of events leading up to and following the immobilization of the ciliate. Slowing first of the somatic ciliature, then

of the oral ciliature was followed by total immobilization. A conspicuous exudate was observed during the slowing process; it was located on the end of cilia and a portion oftentimes was trailing behind the organism. It was presumed that that antigen-antibody combination in some manner influenced ciliary movement. The exudate caused a mechanical slowing of the cilia, only after the initial slowing had taken place. Total immobilization was followed by lysis of the cell.

Study V was an investigation of particular events leading up to lysis of the total immobilization of cilia, using the phase microscope as the tool in this study. In a few organisms a slight movement of the oral cilia was observed within three minutes after total immobilization had occurred. Rupture of the membrane at the oral region immediately followed this "latent" ciliar movement.

II. CONCLUSION

The five studies indicated a significant difference between the activities of B. undulans in normal and immune serum. The immune sera contained a specific antibody which reacted with the antigen substance, B. undulans. The immobilizing antigen may have been a ciliary and/or body antigen. Exudate droplets observed on cilia and the pellicle would provide some evidence of a surface reaction between antigen and antibody. Lysis following immobilization was attributed to a disturbance in the normal external state of

the protozoan (loss of ciliary activity) resulting in a disruption of homeostasis in the interior of the cell. This disruption may have been further indicated by the increase in cytoplasmic flow observed.

This investigation of the antigen-antibody response, using Blepharisma undulans as the test organism supports work done by others who used such protozoa as Tetrahymena pyriformis, various species of Paramecium, and some amebae.

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